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Evaluation of 'Bacteroides' as Indicator Bacteria in Drinking Water

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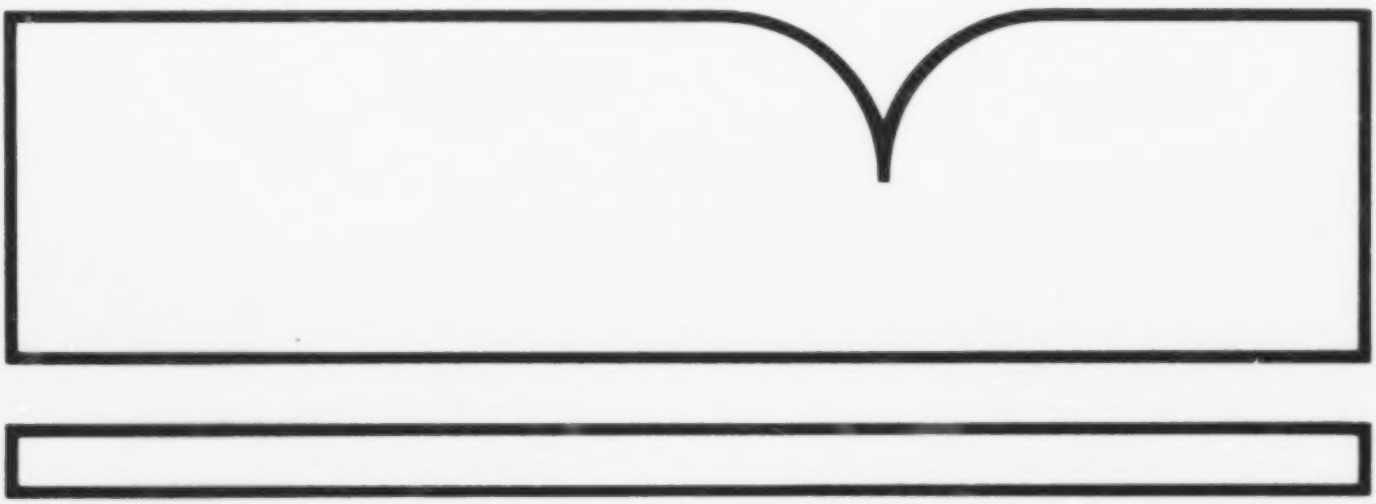

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EVALUATION OF BACTEROIDES AS INDICATOR BACTERIA IN DRINKING WATER

by

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16. ABSTRACT

The research project is concerned with the development of more rapid and sensitive procedures to determine the quality of drinking water. The specific objective of the project was to evaluate the use of a new group of indicator bacteria for assessing fecal contamination of drinking water. The bacteria considered for this study are the human intestinal species of the genus of anaerobic bacteria, Bacteroides. These occur in much higher concentrations in the human than do the coliform bacteria that are currently used as indicator organisms, and they can be detected by serological procedures that are amenable to rapid and sensitive assay. Thus this group appears to offer an ideal alternative to current methodology.

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FOREWORD

The U.S. Environmental Protection Agency is charged by Congress with protecting the Nation's land, air, and water systems. Under a mandate of national environmental laws, the agency strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. The Clean Water Act, the Safe Drinking Water Act, and the Toxic Substances Control Act are three of the major congressional laws that provide the framework for restoring and maintaining the integrity of our Nation's water, for preserving and enhancing the water we drink, and for protecting the environment from toxic substances. These laws direct the EPA to perform research to define our environmental problems, measure the impacts, and search for solutions.

The Water Engineering Research Laboratory is that component of EPA's Research and Development program concerned with preventing, treating, and managing municipal and industrial wastewater discharges; establishing practices to control and remove contaminants from drinking water and to prevent its deterioration during storage and distribution; and assessing the nature and controllability of releases of toxic substances to the air, water, and land from manufacturing processes and subsequent product uses. This publication is one of the products of that research and provides a vital communication link between the researcher and the user community.

This research project is concerned with the development of more rapid and sensitive procedures to determine the quality of drinking water. The specific objective of the project was to evaluate the use of a new group of indicator bacteria for assessing fecal contamination of drinking water. The bacteria considered for this study are the human intestinal species of the genus of anaerobic bacteria, Bacteroides. These occur in much higher concentrations in the human than do the coliform bacteria that are currently used as indicator organisms, and they can be detected by serological procedures that are amenable to rapid and sensitive assay. Thus this group appears to offer an ideal alternative to current methodology.

Francis T. Mayo, Director
Water Engineering Research Laboratory

ABSTRACT

Two immunological techniques and three viable enumeration methods were assessed for their feasibility in detecting intestinal Bacteroides, a potential fecal bacterial indicator group. The two immunological methods, a fluorescent antibody assay (FA) and an enzyme-linked immunosorbentassay (ELISA) were proposed as possible rapid detection methods. For viable enumerations, three selective and differential media were evaluated in the recovery of Bacteroides.

For the ELISA work, Bacteroides fragilis were collected on membrane filters and exposed to rabbit anti-Bacteroides serum (RAB) and goat anti-rabbit conjugated peroxidase (GARP). Kinetic analyses of GARP and colorimetric product absorbance characteristics were found acceptable. However, non-specific binding of GARP to membrane filters occurred. Various attempts were made to minimize the non-specific binding. Filters of differing composition were tested, including polycarbonate, polysulfone, polypropylene, cellulose acetate, cellulose nitrate, and modified polyvinylidene fluoride, and found to be ineffective. Pretreatment of filters with various protein solutions was also ineffective.

Data for the FA procedure were encouraging. The enumeration of organisms in pure culture occurred uniformly and reproducibly. Analyses of raw and primary wastewater indicated ratios of FA positive organisms to fecal coliforms of 600-5000:1, close to the range found in human fecal samples that has been reported in the literature.

Some experiments were performed to simulate contamination of drinking water supplies. This was accomplished by the addition of raw sewage to lake water. Under these conditions it was possible to detect FA Bacteroides spp. using a serum prepared against B. vulgatus, one of the most common species reported from the human intestinal tract. The fluorescence produced was not as intense as with pure cultures, however--partly because of strain specificity and the problem of large amounts of detritus that obscured stained cells and caused non-specific fluorescence.

The survival of viable Bacteroides spp. was also studied using pure cultures and seeding experiments in which washed suspensions of B. fragilis and B. vulgatus were added to the simulated contaminated drinking source water. The viability of Bacteroides spp. decreased rapidly in the seeding experiments so that fewer than 20% persisted after 24 to 72 h. Unseeded, simulated contaminated drinking water contained relatively few obligately anaerobic bacteria. Most anaerobes recovered were facultative anaerobes able to grow aerobically. This result was obtained using all three of the

media designed for the enumeration of viable Bacteroides spp. from natural samples.

Intestinal species of Bacteroides are a potentially useful fecal bacterial indicator group. The results of this study show that they can be detected in wastewaters and simulated contaminated drinking waters using fluorescent antibody microscopic techniques. Futhermore, they can be detected by this microscopic procedure even after their viable numbers have declined precipitously in pure cultures and seeded samples. Using this procedure the intensity of fluorescence of natural samples was lower than with pure cultures. This was due in part to debris accumulation and non-specific staining of debris and possibly also to problems of strain specificity of the fluorescent antiserum. It seems reasonable that the preparation of an appropriate polyvalent fluorescent antiserum might overcome some of this difficulty.

Considerable difficulty was encountered in developing an ELISA procedure for the detection of these bacteria. The primary problem was due to the non-specific adsorption of the goat anti-rabbit peroxidase to membrane filters. This problem was not overcome in this study.

Viable procedures for the enumeration of intestinal Bacteroides spp. from the environment need more developmental work. Currently available procedures are non-selective for the group. It was noted that this group dies off much more quickly than fecal coliform bacteria. Thus, the ratio of intestinal Bacteroides to fecal coliforms may serve a useful purpose. If the ratio were high it would suggest recent contamination of a water supply; if low, it would suggest an earlier contamination event.

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SECTION 1

INTRODUCTION

Water quality testing standards currently in use are based on the enumeration of coliform bacteria and heterotrophic plate count bacteria (3). Because these procedures require incubation periods of 24 hours or more they are not of use in the rapid identification of potential water quality problems. The development of more rapid and sensitive methods to ascertain water quality would permit fecal contamination to be more quickly detected and subsequent corrective actions to be taken more promptly. In this study we investigate the potential applicability of intestinal Bacteroides as alternative indicator bacteria for the detection of fecal contamination using microbiological and immunological techniques.

Intestinal Bacteroides would appear to be potentially useful as fecal indicator organisms because they occur in significantly higher concentrations than Escherichia coli in the human intestinal tract. Slanetz and Bartley (26) found that E. coli was present in human feces at a concentration level of 1.9×10^7 bacteria per gram of fecal dry matter. Moore and Holdeman (19) reported somewhat higher concentrations (2.76×10^8). The Bacteroides fragilis group now comprised of the species B. vulgatus, B. thetaiotaomicron, B. distasonis, B. ovatus, and B. fragilis constitute 20-30% of cultivatable fecal organisms having counts approximating 1×10^{11} cells per gram of dry fecal matter (12,19). This ratio of Bacteroides to E. coli at 100-1,000:1 suggests the potentially greater sensitivity of Bacteroides spp. as fecal indicators in comparison to the coliforms.

The suggestion to use intestinal Bacteroides as a fecal pollution indicator dates back to 1967 when Post, et al. (21) proposed a simple selective isolation medium for the detection of Bacteroides in sewage. Subsequent selective media and identification tests for the Bacteroides group have been presented for both clinical and wastewater systems (1,6,10,20,21,30). In 1979 Hsu, et al. (14) developed a rapid immunoenzyme method for the identification of B. fragilis and Fiksdal, et al. (10) subsequently investigated Bacteroides species as prospective indicator bacteria using viable and immunological enumeration methods.

In this investigation we set out to enumerate presumptive Bacteroides organisms in samples of potable and recreational waters using fluorescent antibody enumeration (FA), enzyme linked immunosorbent assay (ELISA), and viable recovery. A number of different questions were entertained. 1) How effective and sensitive is fluorescent antibody labeling for enumerating

Bacteroides spp. in aqueous environments? 2) How rapid and sensitive is ELISA enumeration of these organisms in aqueous environments? 3) How does the loss of viability of *B. vulgatus* and *B. fragilis* compare with the loss of their avidity for fluorescent antibody? 4) How does the recovery of viable *Bacteroides* of intestinal origin (i.e., intestinal *Bacteroides*) on selective media compare with the recovery of total and fecal coliforms under conditions similar to drinking water becoming contaminated with raw sewage? 5) Which selective media are efficacious in the selection and identification of intestinal *Bacteroides* from aqueous environments?

SECTION 2

CONCLUSIONS AND RECOMMENDATIONS

1. Development of a rapid, fluorescent antibody enumeration procedure.

The development of a fluorescent antibody staining procedure for the detection of intestinal Bacteroides spp. in drinking waters appears promising. However, in this investigation problems were encountered with background fluorescence of particulates in natural samples, debris-coating of Bacteroides cells, and high specificity of available antisera. Nonetheless, if a direct microscopic enumeration procedure for Bacteroides was considered worthwhile, it should be possible to overcome these problems and develop a rapid (albeit somewhat tedious) procedure for enumeration of this group.

2. Development of a rapid, immuno-enzyme enumeration procedure.

It is obvious that the same drawbacks of the immuno-fluorescent technique are inherent in any immuno-enzyme procedure: accumulation of debris during filtration and non-specific binding of antibodies to the membrane filter and debris. In addition, the immunoassay appears less feasible because the relationship of enzyme product to the absolute level of cell antigen is a kinetic relationship subject to the additional factors such as temperature, pH, substrate concentration, and inhibitors. Further investigation into this enumeration approach is not recommended until the problem of non-specific binding of antisera to the filter surface is resolved.

3. Development of a viable enumeration procedure.

Intestinal Bacteroides appear to die off much more quickly than coliform bacteria in natural waters. The significantly greater numbers of Bacteroides in the intestine compared with the representative coliform, E. coli, the demonstrated recovery of Bacteroides from seeded waters, and their rapid decline in viability suggest the further development of a viable enumeration procedure to be used in conjunction with a standard fecal contamination measurement. For example, ratios where Bacteroides outnumber fecal coliforms would suggest recent contamination. Ratios where fecal coliforms are high relative to Bacteroides would indicate contamination > 24 hours old. Further investigation of this correlative relationship would require the formulation and testing of intestinal Bacteroides recovery media which are more efficacious and more selective with respect to facultative anaerobes.

SECTION 3

MATERIALS AND METHODS

Bacterial Media and Strains. The formulations for Brain Heart Infusion (BHI), Wilkins and Chalgren (WC) (1), Bacteroides Bile Esculin (BBE) (16), Kanamycin Bile (KB), and R2A (22) media are shown in Tables 1-4.

Table 1. Brain Heart Infusion Based Media

A.	Base Medium = BHI	per liter
	Brain Heart Infusion	37 g
	Yeast Extract	5.0
	Cysteine $HCl \cdot H_2O$	0.5
	Hemin	10 ml*
	Vitamin K_1	0.2 ml*
	Distilled H_2O	1000 ml
	Agar	(25g)
B.	BHI-Thioglycollate	
	BHI Base	1000 ml
	Methylene Blue	0.4 ml
	Sodium Thioglycollate	0.2 g
C.	Bacteroides Oxgall Esculin (BOE)	
	BHI Base	1000 ml
	Oxgall	20 g
	Esculin	5 g
	Ferric Ammonium Citrate	1
D.	Kanamycin Bile Medium	
	BHI Base	1000 ml
	Oxgall	20 g
	Esculin	5
	Ferric Ammonium Citrate	1
Autoclave and add aseptically:		
	Gentamicin Sulfate	50 mg
	Kanamycin	250 mg
	Penicillin-G	500 units

Table 1. Brain Heart Infusion Based Media (Continued)

E. 20% Bile Salts	
BHI Base	1000 ml
Bile Salts (Difco)	200 g
pH 7.0	
F. 20% Oxgall	
BHI Base	1000 ml
Oxgall (Difco)	200 g
pH 7.0	

*Hemin - dissolve 50 mg in 1 ml 1N NaOH. Make to 100 ml with distilled H_2O. Autoclave at 121°C for 15 min. Vitamin K_1 - dissolve 0.15 ml of Vit. K_1 in 30 ml of 95% ethanol. (Final $[K_1]$ = 1 nl/ml ≈ 1.0 µg 1 ml). Keep refrigerated; discard after one month.

Table 2. Bacteroides Bile Esculin Agar

	per liter
Oxgall (Difco)	20 g
Trypticase Soy Agar	40
Esculin	1
Ferric Ammonium Citrate	0.5
Distilled H_2O	1000 ml
When dissolved add:	
Hemin	20 ml
Gentamicin	100 mg
Adjust to pH 7.0	

Table 3. Wilkins and Chalgren Agar

	per liter
Trypticase (BBL)	10 g
Gelysate (BBL)	10
Yeast Extract	5
Glucose	1
Pyruvate	1
Arginine	1
NaCl	5
Esculin	1
Ferric Ammonium Citrate	0.5
Hemin	10 ml
Vit. K_1	0.2 ml
Agar	15 g
Distilled H_2O	1000 ml
Autoclave and add aseptically:	
Penicillin-G	500 units
Gentamicin Sulfate	50 mg

Table 4. Composition of R2A

Ingredient	per liter
Yeast Extract	0.5 g
Proteose Peptone No. 3	0.5
Casamino Acids	0.5
Glucose	0.5
Soluble Starch	0.5
Sodium Pyruvate	0.3
K_2HPO_4	0.05
$MgSO_4 \cdot 7H_2O$	15.0
Distilled H_2O	1000 ml

Final pH 7.2, adjust with crystalline K_2HPO_4 or KH_2PO_4 before adding agar. Add agar, heat medium to boiling to dissolve agar and autoclave for 15 min. at 121°C, 15 psi.

Intestinal Bacteroides spp. (Table 15) were obtained from Dr. John Johnson, Virginia Polytechnic Institute and State University, and Dr. Barbara Minshew at the University of Washington, and the enterics (Table 15) from Mary Bicknell, Department of Microbiology, University of Washington.

Bacteroides vulgatus. B2-4 was grown in Brain Heart Infusion (BHI) Thioglycollate medium. The medium was boiled and tubed while hot in an anaerobic glove bag (Coy Manufacturing, Ann Arbor, Michigan) and 10 ml volumes distributed into pressure tubes which were sealed with rubber bungs and aluminum crimp tops. The glove bag contained a gas mixture of 10% hydrogen, 5% carbon dioxide and 85% nitrogen. Medium was sterilized by autoclaving at 121°C for 15 min. The culture tubes were inoculated with 0.1 ml of a suspension of B. vulgatus B2-4, and incubated at 37°C without shaking. Cell densities were estimated using a Spectronic 20 colorimeter (Bausch and Lomb) at 660 nm. Viable count spread plates on BHI Agar were performed by plating dilutions of the growth culture and incubating at 30°C or 37°C anaerobically in BBL Gas Pak (Baltimore Biological Laboratories, Cockeysville, MD) containers.

Stationary phase (48 h) cultures of B. fragilis, grown anaerobically in Brain Heart Infusion medium at 37°C in BBL Gas Pak containers, were washed by centrifugation, resuspended in phosphate buffered saline, pH 7.2, and when required, preserved with the addition of formalin to 0.1%. The phosphate buffered saline (PBS) contained 0.01 M KH_2PO_4 and 0.15 M NaCl. PBS with Tween 20 (PBST) was formulated by adding Tween 20 to PBS to a final concentration of 0.05%.

Antisera. Rabbit anti-Bacteroides (RAB) non-labeled sera for B. fragilis and B. vulgatus were obtained from Dr. Barbara Minschew, Department of Microbiology, University of Washington. The Fluoretec-F Test serum (F-RAB) was obtained from General Diagnostics (Morris, Plains, NJ) before being discontinued. This serum is a pooled serum containing antibodies to several species of Bacteroides (i.e., B. fragilis, B. thetaiotaomicron,

B. vulgatus, B. ovatus, and B. distasonis). Goat anti-rabbit serum conjugated to fluorescein isothiocyanate (GARF) or horseradish peroxidase (GARP) and goat normal serum (GNS) were obtained from Miles Laboratories, Inc. and diluted with PBS for use. All antisera dilutions were preserved with 0.01% merthiolate.

Membrane Filters. Membrane filter compositions and pore sizes were polysulfone (Gelman HT-450), 0.45 µm; polypropylene (Gelman), 10 µm; polycarbonate (Nuclepore), 0.2 µm; modified polyvinylidine-fluoride (Millipore-Durapor), 0.45 µm; cellulose acetate (Gelman), 0.45 µm; cellulose nitrate (Whatman), 0.45 µm; Teflon 450 (Gelman), 0.45 µm. Filter diameters were 25 mm.

Bacterial Enumerations. A comparison of various enumeration methods was made. Forty-eight-hour cultures of B. fragilis grown anaerobically at 37°C in Brain-Heart Infusion medium in BBL Gas Pak containers were diluted in sterile phosphate buffered water under laboratory atmosphere. Direct fluorescent antibody (dFA) counts were made by collecting cells on 0.2 µm pore size Nuclepore filters pre-stained with Irgalen Black (to reduce background fluorescence), washing three times with PBS, and adding 1.0 ml of filter-sterilized (0.2 µm) gelatin solution for 20 min. This was followed by washing the filter 2 times with PBS, drying filter on glass slide for about 10 min, reacting with fluorescent antibody for 15-30 min in the dark in a Petri dish moisture chamber at room temperature, vacuum drying excess moisture, washing two times with PBS, drying the slide in the dark, and suspending the preparation in Bacto FA Mounting Fluid, pH 9 (Difco Laboratories, Detroit, MI) before observing under oil immersion using a Zeiss GFL microscope equipped with a Zeiss epifluorescence attachment. Using this protocol rhodamine isothiocyanate was not necessary to reduce background fluorescence. Direct microscopic fluorescent counts were made in the same fashion except acridine orange (AO) was used to stain cells. Total cell counts were performed using a Petroff-Hauser counting chamber with a Zeiss Photomicroscope using a 40X phase objective lens. Viable plate counts for pure culture survival studies were made with Brain Heart Infusion agar pour plates incubated anaerobically at 37°C for 48 h. For environmental samples using lake water seeded with raw sewage, spread plates of various media were incubated in the glove bag at 29-30°C for 48-72 h.

The standard filtration procedure using polycarbonate (PC) filters and subsequent anti-sera exposure was as follows: Cell suspensions were filtered by negative pressure through PC filters and washed 3 times with 5 ml of pre-filtered (0.2 µm) PBS (3 x 5 ml) under negative pressure. Filters were exposed to 1 ml of gelatin solution (the gelatin solution consisted of 2% gelatin in PBS, hydrolyzed in the autoclave for 15 min at pH 11, and neutralized before use) for 15 min, then filtered and washed with 3 x 5 ml PBS. Filters were air dried and then exposed to 30 µl of F-RAB or RAB and incubated for 15 min in the dark at room temperature in a petri dish moisture hamber to prevent drying. Filters exposed to RAB were washed by 3 x 5 ml PBS and then exposed to 30 µl of GARP/GARF in an incubation chamber for 15 min in the dark followed by a 3 x 5 ml PBS wash. Modifications in the standard protocol were made when appropriate and are noted in table legends.

Bacto FA Mounting Fluid (Difco Labs, Detroit, MI) was used for all microscopic preparations.

The smear test is similar to the filter procedure except it is performed on a glass side. A loopful of cells was air dried on to a glass microscope slide and then exposed to a drop of 0.05% glutaraldehyde for 10 min to fix cells to the slide surface. Glutaraldehyde was rinsed away with distilled H_2O and the slide blotted dry. RAB or F-RAB (10 µl) was added and incubated for 15 min in a moisture chamber, and then rinsed with distilled water. For RAB treated cells, GARP or GARF (30 µl) was added and incubated for 15 min and then rinsed with distilled H_2O.

Fluorescent labeled cells were enumerated using an epifluorescence microscope. Peroxidase labeled cells were quantified using the following reaction mixture with a 30 min reaction time: 0.5 ml of PBS; 0.5 ml of orthophenylene diamine solution (40 mg in 100 ml of 0.025 M sodium citrate-0.04 M NaH_2PO_4 buffer, pH 5.0), and 30 µl X 3% H_2O_2 (to initiate the reaction). The reaction was stopped with 0.4 ml 2.5 M H_2SO_4. Absorbance was read at 492 nm using a Spectronic 20.

Total and fecal coliform counts were determined using the membrane filter technique in Standard Methods (3) with m-Endo and m-FC media, respectively. Confirmations were made in brilliant green lactose bile broth.

For evaluating ELISA testing procedures, the non-specific binding of GARP to membrane filters was assessed in the following manner: Filters of various chemical compositions were washed by vacuum filtration with 5-30 ml of PBS, pH 7.3, to remove wetting agents. The washed filters were then soaked in protein solutions of either 2% bovine serum albumin, 2% hydrolyzed gelatin, or goat normal serum for 30 min. Pretreated filters were washed with 5 ml of PBS, pH 7.3. One ml of a dilution of GARP was layered on each filter in a filtration set-up and incubated for 30 min. The filter was washed with 30 ml PBS. Untreated filters were exposed to GARP dilutions of 1:10-1:104. Each filter was placed in a 3 x 10 mm tissue culture dish and peroxidase activity assayed.

Initial experiments to examine recovery of pure cultures of Bacteroides fragilis from tap water and wastewater were performed in the laboratory. In these experiments 48 h BHI broth cultures of B. fragilis strain 2553 were inoculated and diluted in tap water or primary wastewater. Viable counts were determined by plating dilutions on BHI and dFA counts were determined microscopically. The percent recovery was determined by dividing the dFA count by the viable count and multiplying by 100 (Table 6). For those suspended in primary wastewater, the background fluorescent count was subtracted from the dFA so that only the recovery of the spiked B. fragilis was determined.

To simulate the contamination of a drinking water source with fecal material, raw sewage (obtained from the Renton Wastewater Treatment facility) was added to lake water (ship canal water from Lake Washington). In one experiment three separate vessels were used. One vessel (Vessel No.

1) served as a control and contained only raw wastewater (1 part) and canal water (9 parts). Vessels II and III were replicates and contained in addition to the raw sewage an inoculum of Bacteroides vulgatus strain B2-4 grown anaerobically on BHI medium and washed once in PBS. This species was selected because it is reported as the most numerous one from human feces. These flasks were incubated at 17°C with a reciprocating shaker and sampled at zero time and at daily intervals for five days. Direct fluorescent antibody counts of B. vulgatus were determined with an indirect fluorescence procedure using an antiserum to that species. Viable enumerations of total and fecal coliforms were determined in Vessel 1 and viable enumerations of anaerobes was determined on BBE and WC media incubated in an anaerobic glove bag at 29°C for 48 to 72 h. Data for this experiment are shown in Fig. 1 and Fig. 2A.

In a second simulation experiment, pure cultures of both B. vulgatus and B. fragilis were seeded at similar concentrations into a mixture containing nine parts of canal water and one part of raw sewage. For this experiment a pooled antiserum containing both anti-B. fragilis 2553 and anti-B. vulgatus B2-4 was used (Fig. 4 and Fig. 5).

The two Bacteroides cultures were grown in BHI + 2% Oxgall broth and washed once by centrifugation with PBS. The final concentration was 1.2×10^8 Bacteroides/ml (Fig. 3).

Oxygen in the test vessels was determined using Carpenter's modification of the Winkler method (28). Ship Canal water and the Ship Canal/raw wastewater mixture at time zero and 24 hours were both oxygen saturated.

The Bacteroides recovery and confirmation sequence is shown in Table 16.

SECTION 4

RESULTS

Fluorescent Enumeration. To calibrate our filtration set-up and familiarize ourselves with the direct fluorescent antibody (dFA) procedure, we did a preliminary comparison of methods using pure cultures of B. fragilis as shown in Table 5. Agreement within a factor of 1.1-3.1 was found among methods indicating that the dFA technique is satisfactory for enumerating B. fragilis in aqueous samples.

Table 5. Preliminary comparison of enumeration techniques for pure cultures of B. fragilis.

	Bacterial counts/ml (X 10^9)		
Experiment	Counting Chamber	Viable[a]	dFA[b]
I	1.9	-[c]	1.7
II	2.2	-	1.3
III	2.2	-	1.4
IV	4.4	2.3	1.4
V	-	2.3	1.1

All values are the average of duplicate determinations.

[a] BHI medium used for viable enumeration.

[b] dFA = direct fluorescent antibody assay using Fluoretec-F commercial reagent

[c] = Not enumerated.

A determination of the percent recovery of the dFA technique was made by assessing the presence of B. fragilis in buffer, tap water, and primary wastewater each of which was used to dilute pure cultures of the indicator organism (Table 6). The recovery of the dFA was quite variable. Whereas cells diluted with buffer approached a maximum and total recovery, dilution of the cells by either tap water or primary wastewater resulted in significantly reduced detection of the bacteria in the sample for reasons that are unknown. There was also an apparent quenching of the fluorescence by unknown factors.

The quantification of dFA Bacteroides spp. in raw wastewaters was determined using the polyvalent Fluorotec-F antiserum (Table 7). dFA positive cells were usually but not always detectable in wastewaters and in one comparison exceeded fecal coliform organisms by a factor 1000.

Table 6. Percent recovery of B. fragilis strain 2553 (dFA positive) from seeded tap water and primary wastewater in comparison to viable plating on BHI medium.

Diluent	% Recovery
Phosphate Buffered Water	36-96%[a]
Tap Water	8-32%[b]
Primary wastewater	5-11%[a]

a = Three samples
b = Eight samples

Table 7. Enumeration of fecal coliforms and dFA positive organisms in wastewater using polyvalent Fluorotec-F antiserum.

Source	Fecal Coliforms/100 ml	dFA/100 ml
Raw wastewater	0.3×10^5	400×10^5
Raw wastewater	0.6×10^5	ND[b]
Primary wastewater	-[a]	2900×10^5
Primary wastewater	-	3100×10^5

a Not Enumerated
b ND - Not Detected $\leq 5.8 \times 10^4$/ml

During our investigations the production of the commercial Fluoretec-F reagent used for our direct FA was discontinued. To continue developing a fluorescent labeling technique we began to use an indirect FA technique (iF-A) where an unlabeled antiserum is first exposed to the target cell (i.e., rabbit anti-Bacteroides followed by fluorescein labeled anti-rabbit serum which labels the bound RAB antibodies). As Table 8 shows, double labeled cells with one exception were essentially non-detectable even though directly labeled cells using the commercial fluorescent label occurred. Repeated attempts to achieve fluorescent double labeling were unsuccessful. To assess if our RAB serum was active, various agglutination and fluorescent procedures were tested including hemagglutination plates, glass microscope slides, and small test tubes, all of which were unsuccessful. RAB activity, however, was successfully assessed using a glass slide smear technique using indirect fluorescent labeling. All vials of RAB fragilis sera tested were found to be active with GARF as shown in Table 9.

Table 8. RAB Concentration Dependent iFA Formation[a]

RAB Concentration	Total Fluorescent Cells/ml
No cells - RAB	ND[b]
Cells +	
1X RAB	ND
0.8X RAB	ND
0.6X RAB	ND
0.4X RAB	ND
0.2X RAB	ND
0.1X RAB	2.3×10^8
No RAB	ND
dFA	2.3×10^8

[a] Cells were filtered, washed, gelatin treated for 15 min and exposed to RAB for 30 min followed by PBS wash and 10^{-1} GARF exposure for 30 min and washed.
[b] ND = Not Detected

Table 9. GARF titer determination using smear test

GARF	- Qualitative Labeling by GARF -		
Dilution Factor	RAB No. 1	RAB No. 3	RAB No. 6
1	4+	4+	4+
4	3+	4+	4+
8	3+	3+	4+
16	4+	4+	2+
32	3+	4+	** 4+
64	3+	3+	2+
128	2+	** 4+	1+
256	2+	1+	1+
512	** 3+	2+	1+
1024	1+	1+	0
** Titer	512	128	32

For both B. vulgatus and B. fragilis we tested the reproducibility of collecting and labeling (iFA) cells on membrane filters as shown in Table 10. The percent recovery of iFA labelled cells on filters as compared to direct cell counts was about 63% for B. fragilis and 66% for B. vulgatus.

Table 10. Reproducibility and comparison of iFA enumerations to Petroff Hauser counting chamber total microscopic counts

	Number Cells/ml	
	B. fragilis 2553	B. vulgatus B2-4
i/FA Count	1.7×10^{9}[a]	7.9×10^{8}[a]
Direct Count	2.7×10^{9}[b]	1.2×10^{9}[b]

a = Average of triplicate determinations
b = Average of duplicate determinations

Enzyme-Linked Immunosorbent-Assay. Due to the increased sensitivity the ELISA is purported to possess over FA techniques, we focused our studies on this area. Exposure of goat anti-rabbit peroxidase serum (GARP) to polycarbonate (PC) membrane filters resulted in non-specific binding to the filters as shown in Table 11 as also occurred with the iFA technique. As the concentration of GARP exposed to the filters increased, the peroxidase activity remaining associated with washed filter also increased. Assuming an acceptable background of A_{492} = 0.10, the GARP had to be diluted to a level of 3.3×10^{-6} - 1×10^{-5} before the non-specific binding activity was $\leq A_{492}$ = 0.10.

Table 11. GARP Non-specific binding endpoint dilution[a]

Dilution	Optical Density (492 nm)
No filter-No GARP	0.03
Filter-No GARP	0.03
10^{-6} GARP	0.07
3.3×10^{-6} GARP	0.08
10^{-5} GARP	0.20
3.3×10^{-5} GARP	0.30
10^{-4} GARP	1.2
10^{-3} GARP	1.7

a = 3x5ml PBST wash; 30 min assay

An attempt to wash the GARP from the PC filters is recorded in Table 12. For this experiment Tween 20 was added to PBS to improve washing. Regardless of the number of volumes or the total volume of PBS passed through the filter, the GARP exhibited a fairly constant minimum level of binding. In addition, that level appeared dependent on the initial GARP concentration exposed to the filter.

Table 12. GARP removal through filtration washing[a]

Dilution	PBST Wash	Optical Density (492 nm)
10^{-4} GARP	3 x 5 ml	0.14
	6 x 5	0.16
10^{-3} GARP	3 x 5	0.88
	6 x 5	0.85
	3 x 10	1.2
	4 x 10	0.80
	5 x 10	1.4
	6 x 10	0.80

[a] = Cell concentration $2x10^8$/ml-filter; No RAB; 30 min assay

To keep the non-specific peroxidase activity background at approximately A_{492}=0.1, we chose a 10^{-5} dilution of GARP for our initial ELISA tests. To establish if GARP non-specifically bound to B. fragilis cells we exposed varying cell concentrations trapped on PC membranes to 10^{-5} GARP as shown in Table 13. There appeared to be no cell dependent increase in GARP activity.

Table 13. Non-specific binding of GARP to filtered cells[a]

Cell Concentration	Optical Density (492 nm)
10^8/ml - No GARP	0.01
10^8 + GARP	0.04
10^7 + GARP	0.04
10^6 + GARP	0.04
10^5 + GARP	0.03
10^4 + GARP	0.04
No cells + GARP	0.10

[a] = 10^{-5} GARP with 15 min exposure; 3x5ml PBST wash; 15 min assay

Similarly, since RAB is the second component in the ELISA double conjugate system we exposed the filters to RAB and GARP to determine if the level of non-specific binding was increased. Table 14 indicates that prior exposure of filters to RAB under these conditions showed only a slight increase in non-specific binding with increasing RAB concentration.

Table 14. Non-specific binding of RAB:GARP conjugate to PC filters[a]

RAB Dilution	Optical Density (492 nm)
- RAB, - GARP	0.04
- RAB, + GARP	0.05
10^{-2} RAB, + GARP	0.05
2×10^{-2} RAB, + GARP	0.08
3.3×10^{-2} RAB, + GARP	0.05
5×10^{-2} RAB, + GARP	0.09
10^{-1} RAB, + GARP	0.10

[a] = 15 min RAB exposure; 10^{-5} GARP 15 min exposure; 3x5ml PBS wash; 30 min assay

Preliminary attempts to show a cell dependent peroxidase activity increase using the indirect double conjugate assay (Cell:RAB:GARP) were unsuccessful. Increasing the exposure time of RAB to cells on PC filters from 15 min to 7.5 h and of GARP to RAB from 15 min to 17 h showed no significant increase in color development.

We concluded from these data that the greatest impediment to the success of developing an ELISA procedure using membrane filters was the non-specific binding of antibodies to the filter itself resulting in unacceptably high background levels of enzyme activity. To minimize non-specific binding we assessed a variety of membrane filters of varying composition for their non-specific binding capacities. In addition, we pretreated the filters with 2% gelatin, 2% bovine serum albumen, and goat normal serum (12.4% protein) to competitively bind all non-specific binding sites. Neither the composition of the filters (i.e., cellulose nitrate, polyvinylidine fluoride, polysulfone, polycarbonate, cellulose nitrate or teflon) nor the pretreatment of the filters with protein-containing solutions resulted in acceptable background enzyme levels (i.e., A_{492} enzymatic color formation <1.0).

Viable and iFA Enumerations of Bacteroides in Natural Waters. Based on the results of our initial data and techniques we decided to enumerate intestinal Bacteroides in receiving waters by focusing on both indirect fluorescent labeling of cells and the recovery of viable organisms on selective indicator media. Since B. vulgatus is the most prevalent organism in human feces we performed a growth curve and specifically looked at the relationship of viability to other enumeration procedures (Fig. 1). Direct counts, absorbance, and iFA counts remained stable over time while viable counts declined 740-fold over eight days.

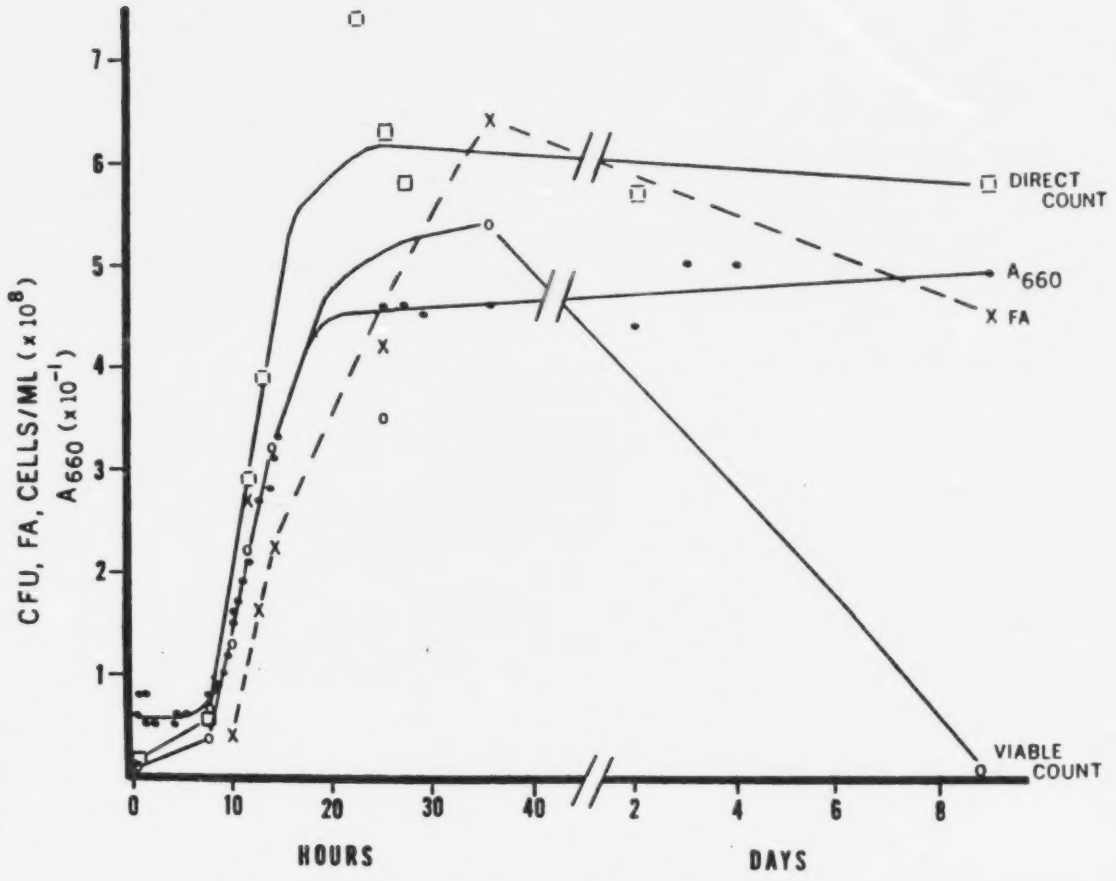

Fig. 1. Growth curve of Bacteroides vulgatus B2-4 showing the comparison of direct counts, A_{660}, fluorescent enumeration, and viable counts.

The confirmation criteria used to distinguish viable intestinal enteric Bacteroides are esculin hydrolysis, kanamycin and penicillin resistance, erythromycin sensitivity, and growth in 20% bile salts medium (BHI). We

tested our stock organisms to establish their responses to the different confirmation criteria. The results are shown in Table 15. Of particular interest is that not all the enteric Bacteroides were positive for all the criteria. B. fragilis 2393 and B. vulgatus were penicillin sensitive, and none of the strains grew on 20% bile salts BHI.

Table 15. Confirmation characteristics for stock strains

STRAIN	Esculin Hydrolysis	Kanamycin Resistance (1000 µg)	Penicillin Resistance (2 units)	Erythromycin Resistance (60 µg)	20% Bile Salts Growth	20% Oxgall Growth
B. fragilis						
VPI 2393	+	+	-	+	-	-
VPI 2553	+	+	+	+	-	-
B. thetaiotamicron						
VPI 5482	+	+	+	+	-	±[a]
VPI 2302	+	+	+	+	-	±
B. distasonis						
VPI 4243	+	+	+	ND[b]	-	-
VPI 0052	-	+	+	ND	-	-
B. vulgatus VPI B2-4	-	+	-	+	-	-
B. ovatus VPI 0038	+	+	+	+	-	-
Klebsiella sp.	+	-	+	-	-	±
E. coli 365	-	-	+	+	-	+[c]
E. aerogenes	-	-	+	-	±	+

a ± = Growth on agar/no growth in broth
b ND = Not determined
c + = Growth on agar and broth

Seeding experiments were performed to simulate the contamination of a drinking water source with fecal material. Lake Washington ship canal water was chosen as the water source and nine parts of this were mixed with one part of raw sewage from the Renton Wastewater Treatment facility.

In the first seeding experiment one vessel of this lake water-raw sewage mixture was inoculated with a PBS-washed cell suspension of Bacteroides vulgatus to give an initial concentration of 5.7×10^7 cells per ml. The survival of this culture was assessed by performing iFA counts using an anti-B. vulgatus serum and conducting viable counts on two selective media, BBE (1) and WC (2), that have been used for growth of intestinal Bacteroides spp. The results in this first vessel are shown in Figure 2. Note that the initial iFA counts of B. vulgatus were somewhat below 10^7 per ml (the control vessel containing only the raw sewage and lake water had initial iFA of B. vulgatus of 1.1×10^5, well below that of the vessel inoculated with

B. vulgatus). The iFA counts of this species remained above 10^6 per ml for two days and on day 3 declined to a level just above that of the zero time background (i.e., about 10^5 per ml). In addition, there was interference with background debris on day 4 which made it difficult to detect fluorescence so counts after that could not be accurately assessed. Viable counts of this species were best performed on the WC medium which recovered 19% of the initial inoculum at zero time whereas BBE recovered only 1%. The rate of die-off on the WC medium was logarithmic and paralleled that of the loss of iFA fluorescence.

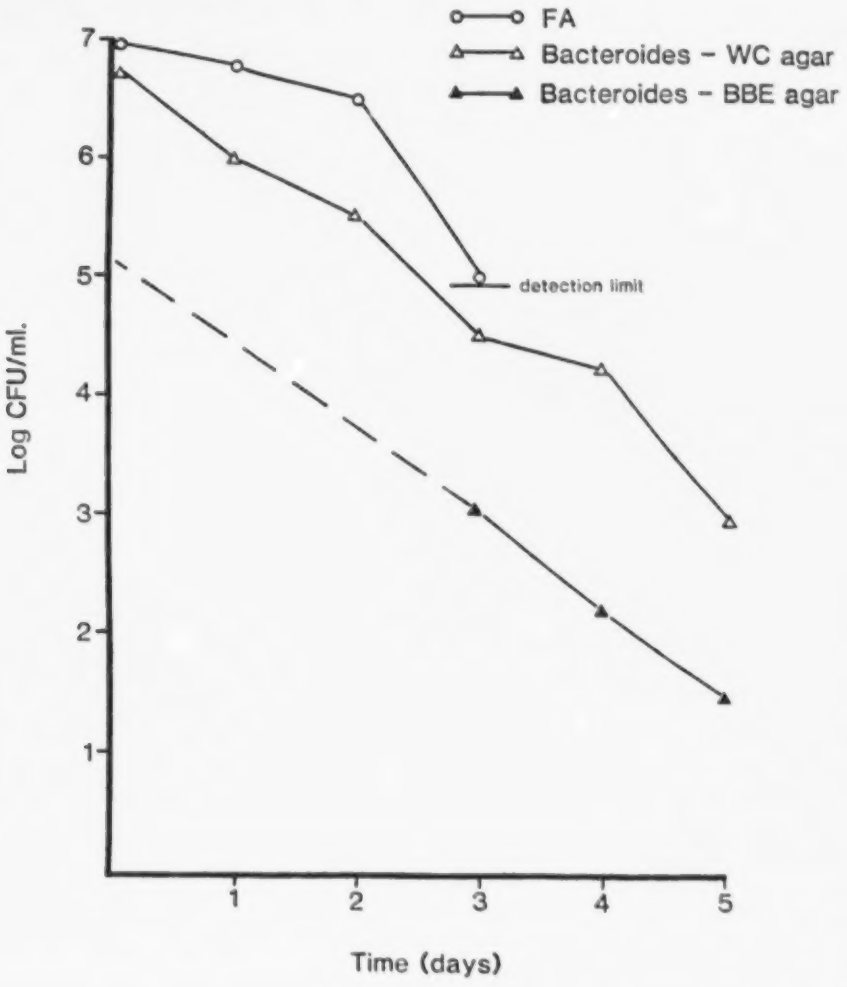

Fig. 2. A vessel containing Lake Washington Ship Canal water and raw sewage (9 parts canal water and 1 part sewage) was seeded with a pure culture of washed B. vulgatus cells. Viable and fluorescent antibody counts for Bacteroides spp. were made at daily intervals. November 14, 1984. All numbers are averages of triplicate determinations.

In the control flask which received only raw sewage and lake water (no B. vulgatus) counts were made of anaerobic bacteria on BBE agar and WC agar and total and fecal coliform bacteria were also enumerated (Figure 3). In addition iFA counts were also performed for B. vulgatus (data not shown).

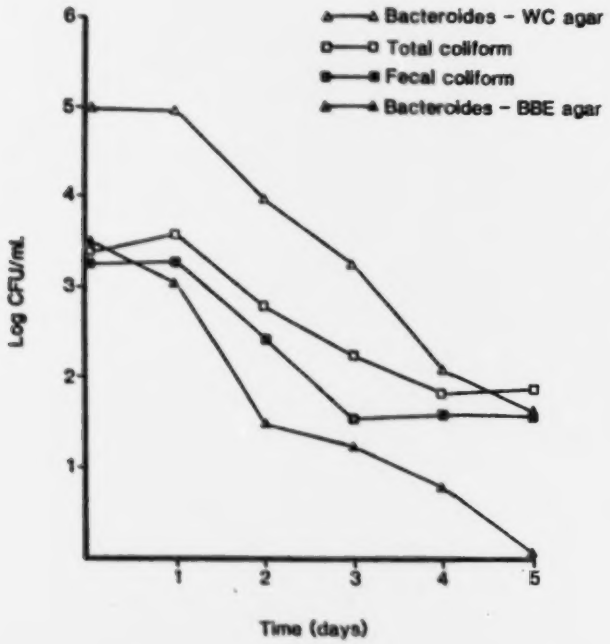

Fig. 3. Viable numbers of various indicator bacteria were determined at daily intervals after simulating the contamination of a drinking water source (Lake Washington Ship Canal water) with a raw sewage sample by mixing 9 part canal water to 1 part raw sewage. This vessel was not seeded with pure cultures of Bacteroides spp. November 14, 1984. All numbers are averages of triplicate determinations.

The iFA counts per ml at zero time were 1.1×10^5 and declined at day one to 3.5×10^4 and at day 2 to 3.1×10^4 and at day 3 to 2×10^3 after which they could not be counted because of interference by debris. Viable bacterial concentrations remained high for one day, then began declining on all media between one and three days. WC medium was superior to the BBE medium for recovery of anaerobic bacteria. Indeed, the counts on this medium closely paralleled the iFA counts of B. vulgatus. However, further testing using colonies randomly picked from these two media and criteria to presumptively identify Bacteroides spp. (Figure 4) showed that many of these anaerobes were not Bacteroides spp. For example, subsequent plating of colonies from WC and BBE media indicated that only 41% of the WC isolates and 76% of the BBE isolates were truly obligate (i.e., non-facultative) anaerobes (Table 16, Exp. 1). We also noticed that many of the facultative anaerobic organisms as well as the anaerobes were esculin hydrolysis positive.

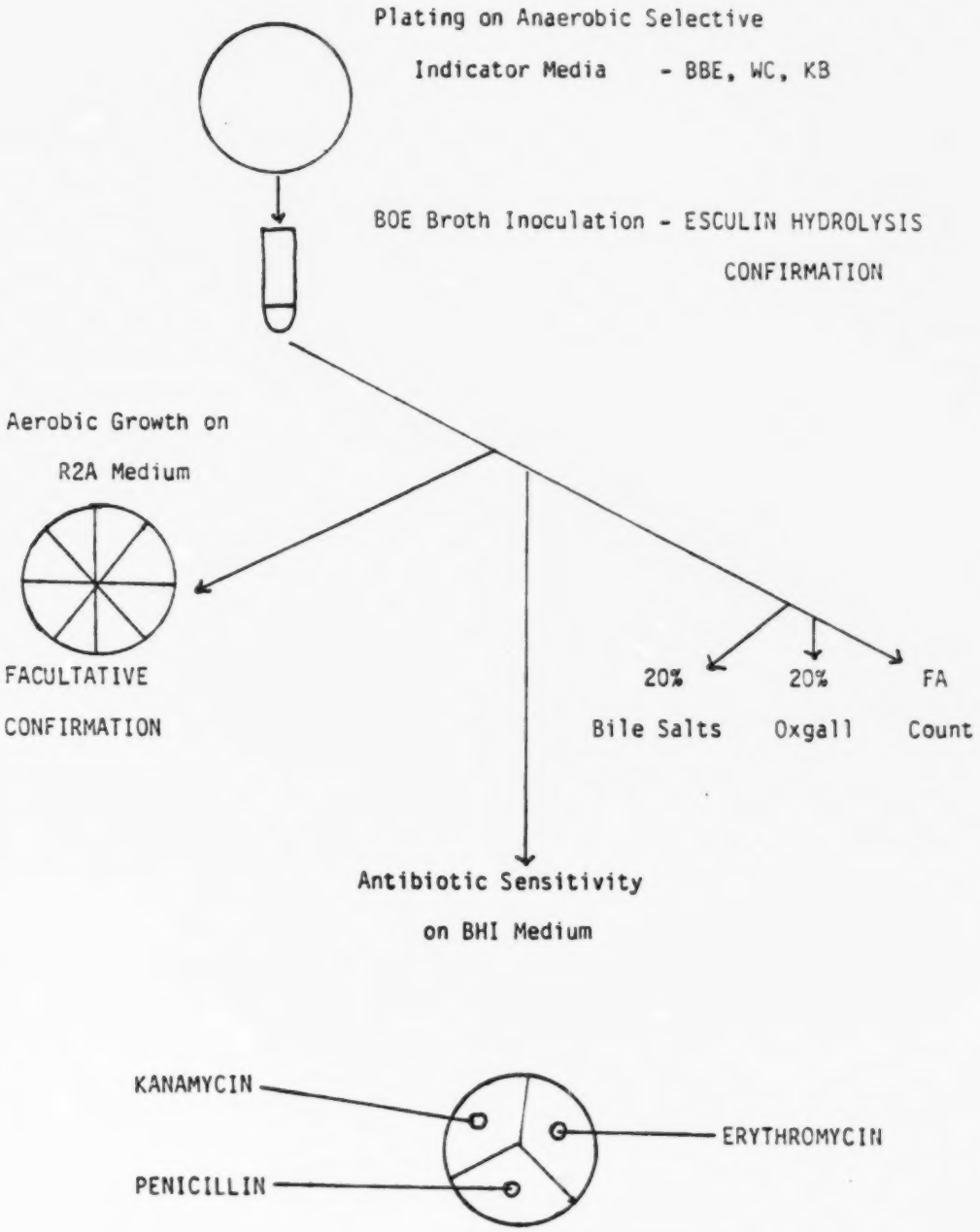

Figure 4. Recovery and confirmation flowchart for intestinal Bacteroides

Table 16. Compilation of confirmation characteristics for selected isolates from recovery media

Characteristic	Recovery Media WC	BBE	KB
EXPERIMENT I - VESSEL I			
1. Total Isolates	51	45	-
2. Total Anaerobes	21 (41%)	34 (76%)	-
3. Esculin Hydrolysis	26	25	-
4. Kanamycin Resistance	20	35	-
5. Penicillin Resistance	36	36	-
6. Erythromicin Sensitivity	38	27	-
7. Characteristics 2-6	1 (2%)	10 (22%)	-
8. *Bacteroides*-like*	17 (33%)	22 (49%)	-
EXPERIMENT II - VESSEL I			
1. Total Isolates	36	38	39
2. Total Anaerobes	16 (44%)	5 (13%)	2 (5%)
3. Esculin Hydrolysis	18	23	25
4. *Bacteroides*-like*	7 (19%)	2 (5%)	2 (5%)

* Gram-negative obligate anaerobes that are both kanamycin resistant and erythromycin sensitive

Evaluation of the antibiotic sensitivities indicated many of the facultative anaerobic organisms had sensitivity patterns similar to the intestinal *Bacteroides*. When the characteristics were grouped for *Bacteroides* confirmation, i.e. esculin hydrolysis, resistance to kanamycin and penicillin, sensitivity to erythromycin, and anaerobiasis, only 22% of the BBE isolates and 2% of the WC isolates could be confirmed.

As we noted, however, not all our stock *Bacteroides* spp. hydrolyzed esculin or were sensitive to penicillin, so a second grouping was made consisting of obligate anaerobes, kanamycin resistance and erythromycin sensitivity i.e. "*Bacteroides*-like." Thirty-three percent of the WC isolates and 49% of the BBE isolates were "*Bacteroides*-like."

In the second seeding experiment, the same protocol was followed except that one vessel (Vessel II) received a PBS washed cell suspension of two *Bacteroides* spp., *B. vulgatus* B2-4 and *B. fragilis* VPI 2553 each at approximately equal concentrations to give a final concentration of about 10^7 per ml. Furthermore, a pooled antiserum to both species was used in the iFA testing of *Bacteroides* spp. in this experiment. The results for Vessel I are shown in Figure 5 and for Vessel II in Figure 6.

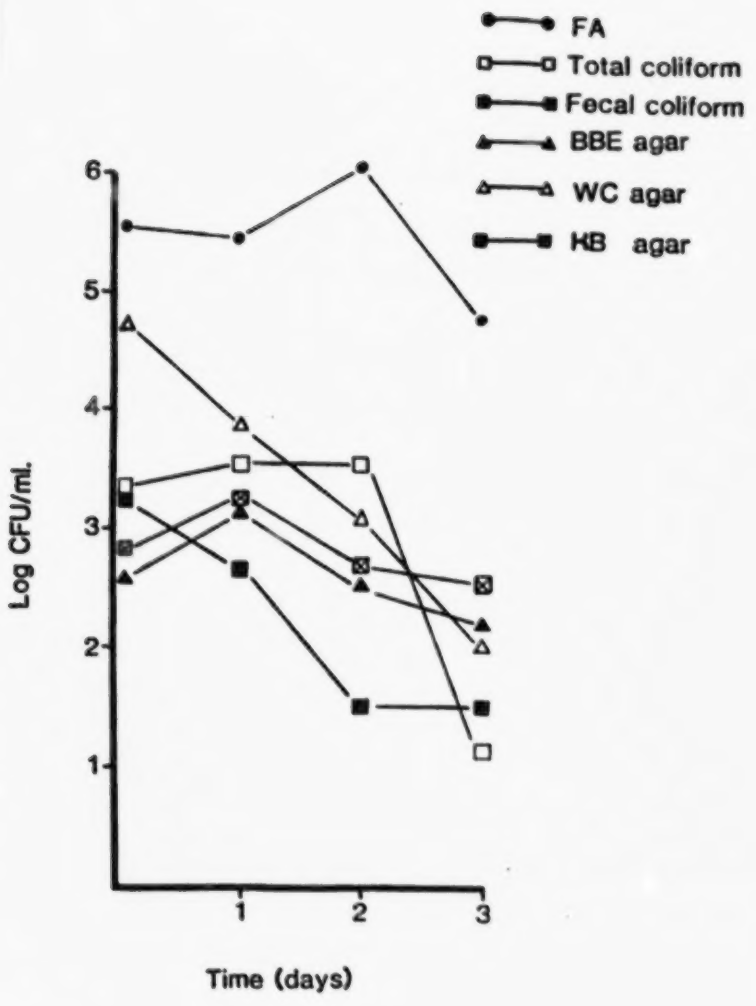

Fig. 5. Viable numbers of various indicator bacteria and fluorescent counts of Bacteroides spp. after simulating the contamination of a drinking water source (Lake Washington Ship Canal water) with a raw sewage sample by mixing 9 parts canal water to 1 part sewage. This vessel was not seeded with pure cultures of Bacteroides spp. December 11, 1984. All numbers are averages of triplicate determinations.

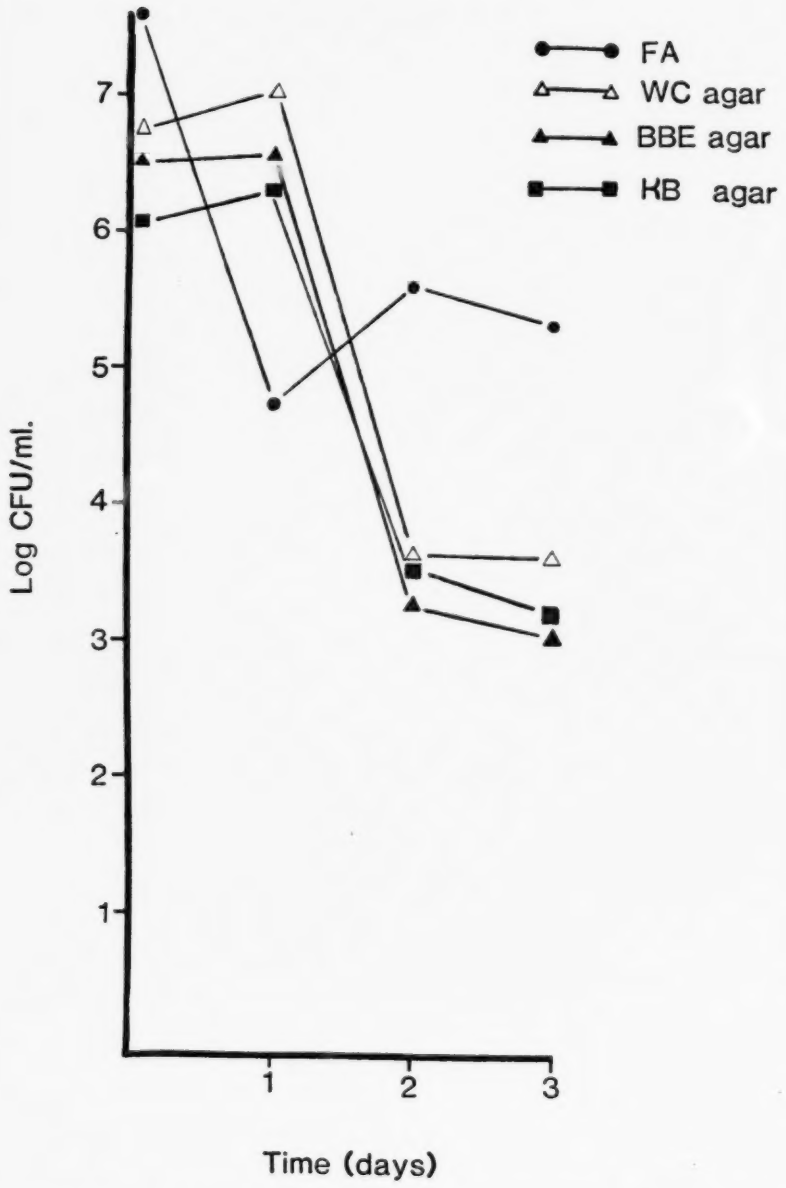

Fig. 6. Anaerobic viable numbers and fluorescent counts of Bacteroides spp. following the seeding of a Lake Washington Ship Canal/Raw sewage mixture (10:1) with a pure culture mixture of B. vulgatus and B. fragilis. December 11, 1984. All numbers are averages of triplicate determinations.

The iFA counts for the control vessel (Fig. 5) were higher than in the previous experiment ranging from 3.6×10^5 initially to a low of 8.3×10^4 after three days. Although, as expected, much higher counts of iFA Bacteroides spp. were noted initially in Vessel II which had been inoculated with the pure cultures of B. fragilis and B. vulgatus, there was a precipitous drop in iFA counts thereafter so that at day 3 there were only about twice as many (i.e., 2.1×10^5) as the control vessel at that time.

The decline in anaerobic organisms was monitored with BBE and WC media plus a newly formulated medium, Kanamycin Bile Medium (KB). This latter medium used BHI as a base and included the selective antibiotics of the WC medium (gentamicin and penicillin-G), 2% Oxgall to enhance the growth of Bacteroides plus 250 mg/l Kanamycin sulfate to which all the Bacteroides reference strains were resistant as well as those unknown anaerobes isolated in Experiment I. The results for the control vessel (Vessel I) are shown in Figure 5. As in Figure 3, the WC medium recovered the highest counts, by nearly 3 logs which steadily declined between days 0-3. Lower counts were seen on the BBE and KB media. Die-off patterns were similar for these two media. Forty-four percent of the isolates from WC medium were obligate anaerobes; 19% of these were Bacteriodes-like (Table 16). Obligate anaerobes constituted only 13% of the isolates from BBE. Only 5% fit the Bacteroides-like grouping. The new selective and differential medium, KB, yielded only 5% anaerobes with 5% Bacteroides-like.

Fecal coliform counts showed a 2 log decrease in two days with no observed decrease between days 2-3. Total coliforms showed no decrease until day three.

For the vessel seeded with raw sewage and Bacteroides cultures (i.e., Vessel II), attempts were made to pick equal numbers of B. vulgatus (esculin -) and B. fragilis (esculin +) colonies (Table 17). First, it was difficult to distinguish one organism from the other because of the weak esculin reaction on these media. Second, it appeared that all the colonies present were B. fragilis. No esculin negative colonies were present. The result is that all the surviving isolates were esculin positive anaerobes, most likely B. fragilis. As the experiment proceeded, the number of anaerobes declined for all the media as also noted in Figure 5, presumably due to the decline in B. fragilis viability. Note, however, that most of the isolates recovered on these plates remained esculin positive (Table 17).

Table 17. Recovery of organisms from seeded Vessel II, Experiment II*

	Total Isolates								
	WC			BBE			KB		
Time(h)	Isolates	Esculin	Anaerobes	Isolates	Esculin	Anaerobes	Isolates	Esculin	Anaerobes
To	7	7	7	10	10	10	10	10	10
T_1 (24h)	8	5	8	6	6	6	8	8	7
T_2 (48h)	10	2	2	9	4	1	9	6	0
T_3 (72h)	7	5	3	10	6	0	9	8	0

*Attempts were made to select 5 esculin positive (i.e. B. fragilis) and 5 esculin negative (i.e. B. vulgatus) from each plate at each time interval.

It is apparent that esculin hydrolysis is not a good differential characteristic to distinguish Bacteroides from other bacteria. The number of esculin positive, non-Bacteroides organisms recovered on the non-selective BOE medium was high (Table 18). Randomly picked esculin positive and negative colonies were assessed for their ability to grow aerobically (Table 19). None of the esculin hydrolysis positive isolates were obligate anaerobes while 2 of the 18 (11%) esculin negative isolates were obligate anaerobes.

Table 18. Non-specific isolation of anaerobically viable organisms on Bacteroides Oxgall Esculin (BOE) Medium

	Esculin Hydrolysis	No Hydrolysis
Total Isolates	47	18
Total Anaerobes	0 (0%)	2 (11%)

Table 19. Growth of isolates on 20% Bile Salts-BHI and 20% Oxgall-BHI

Total Source	Total Isolates	20% Anaerobes	Bile Salts	20% Oxgall
Non Selective BOE Medium	9	0	9	0
Exp. II/Vessel I All Media	56	18	22	1
Bacteroides Stocks	8	8	0	0 (See Table 2)

Growth on 20% bile salts or 20% Oxgall medium is considered to be a confirmatory characteristic for intestinal Bacteroides. None of our stock Bacteroides grew on 20% bile salts and only B. thetaiotamicron grew on 20% Oxgall when it was incorporated in agar (Tables 15 and 19). A simple comparison of growth on bile media was made (Table 11) which indicated that 9/9 aerobic isolates were readily capable of growth in 20% bile salts. Similarly, more isolates from Exp. II/Vessel I were able to grow on 20% bile salts than were anaerobic isolates indicating the charateristic is not by itself useful for selecting Bacteroides. It was also noted that 20% Oxgall is considerably more selective than 20% bile salts.

SECTION 5

DISCUSSION

The use of an indirect Fluorescent Antibody (iFA) technique for the detection of Bacteroides provided a rapid and practical approach to confirming this group in clinical specimens (27). To increase the sensitivity Hsu, et al. (14) utilized an indirect immunoperoxidase (IP) assay that was 91% specific for B. fragilis, in particular, and exhibited a limit of detection of 10^4 cells. The introduction of a commercial direct Fluorescent Antibody (dFA) kit, Fluoretec-F (General Diagnostics, Morris Plains, NJ) (20), was capable of detecting 61-98% of intestinal Bacteroides spp. in clinical specimens using a polyvalent antiserum. Fiksdal, et al. (10) applied this approach to assess Bacteroides as a prospective indicator group for the assessment of fecal contamination. They concluded that although the viability of B. fragilis declines rapidly during the first 24 hours of exposure to aerobic waters, the antigenic presence of the organisms was still detectable after 8 days.

The focus of this study to investigate the findings of Fiksdal, et al. (10) in greater depth began with an assessment of the capacity of the commercial Fluoretec-F to quantify B. fragilis. The dFA enumeration of pure cultures compared favorably with other enumeration techniques (Table 5), while the success at detecting the same species with dFA in potable water or primary wastewater was only 5-32% (Table 6). In spite of low recoveries the potential sensitivity of the assay relative to the fecal coliform indicators was born out when dFA indicated the Bacteroides:fecal coliform ratio was 10^3:1. The discontinuation of production of the commercial antiserum compelled us to look at an indirect Fluorescent Antibody (iFA) assay for future investigations after we attempted several times to conjugate our own fluorescein-labeled antibodies. Using iFA we achieved a reproducibility factor of 63-66% (Table 10).

The ELISA method is theoretically a more sensitive method to detect the antigens of organisms whether viable or not. Thus, we attempted to couple this methodology to membrane filtration technology because of the latter's ease of use, precision and rapidity, and applicability to large volumes needed to assess potable and recreational water quality. Unfortunately, non-specific binding of the goat anti-rabbit peroxidase antiserum to membrane filters resulted in unacceptable background levels and the inability to discriminate background from cell-dependent levels of enzyme product formation. Attempts to minimize this non-specific binding by extensive filter washing, modifications in incubation times, pretreatment of filters with various pro-

tein solutions, and the use of filters of different composition were all unsuccessful. These experiments involved only the use of pure cultures. Further attempts to develop the ELISA approach using membranes to concentrate natural samples would probably encounter greater difficulties such as non-specific binding of antisera to filtered debris as well as the unknown factors which appeared with the low dFA recoveries (Table 6).

Using the iFA technique and various selective-indicator media we assessed the antigenic stability and loss of viability in seeding experiments of two species of Bacteroides (B. fragilis and B. vulgatus) after determining that iFA counting can be reproducible using pure cultures of these two species. In addition, two media, BBE and WC, suggested in the literature (1,16) as potential selective and differential media for the isolation and identification of enteric Bacteroides from sewage or natural waters were used in these studies. In addition, KB medium, a medium of our own formulation was used to follow the presence of viable Bacteroides in our experiments.

Most research on the recovery and differentiation of intestinal Bacteroides has been directed at B. fragilis primarily because this is the species that is most often isolated from clinical specimens (14,23). B. vulgatus, however, is the most prevalent organism in the human intestine while B. fragilis is the 19th most common organism (12,19). We decided to use both B. fragilis and B. vulgatus in our studies to allow comparisons of the best known to the most prevalent species.

B. vulgatus exhibits a typical growth curve on BHI with a doubling time ranging from 1.6-3.2 hours at 37°C without agitation. There is good agreement between direct counts, optical density (A_{660}), and fluorescent enumeration. The die-off in the broth culture indicates that this species may be an indicator of fecal contamination that does not remain culturable in the environment for extended periods of time. Fluorescent counts paralleled viable counts during growth but remained much higher after the viability decreased. This indicates the potential for continual enumeration using iFA counts even after culturability has been lost. The 2 log decline in the iFA counts after eight days may reflect a loss of antigenic integrity of non-viable cells over time.

From the available data it seemed reasonable that the presence of intestinal Bacteroides could be detected in the environment using our iFA technique. We decided to test this assumption and at the same time recover viable Bacteroides using two selective and differential media espoused in the literature, BBC and WC.

The results of the iFA detection were not completely satisfactory. Although iFA counts appeared to follow the levels of viable B. vulgatus (Fig. 2), in Fig. 6 there is little correlation between the viable count curves and the iFA enumerations. The control vessels not seeded with pure cultures also contained fluorescent Bacteroides cells [Fig. 3 (data not shown), and 5]. Although iFA counts were obtained in both experiments, there are certain difficulties with the data. First, when natural waters are filtered through Nuclepore polycarbonate filters they rapidly clog so that

only small volumes are filterable. As more sample is forced through the filter a layering occurs which makes iFA labeling of the trapped cells difficult or impossible. Second, the quality of the labeling is poor with respect to pure culture controls. With pure cultures positive labeling is designated by 3+ or 4+ results on a four point scale whereas with natural samples, the positive samples appear to be 2+ or 3+ and rarely is strong labeling in the 4+ range observed. This suggests there may be problems with serological specificity of strains. Lastly, non-specific labeling of the debris on the filter is another drawback because it reduces the labeling contrast.

In the first seeding experiment B. vulgatus was readily recoverable on BBE and WC from our simulated contaminated drinking source water (Fig. 1). In the second seeding experiment B. fragilis was mixed with B. vulgatus. The numbers of viable cells recovered was comparable across the three different media (Fig. 6). There was an indication that these isolates were mostly B. fragilis which is a hardier species than B. vulgatus.

The survivability of B. fragilis over B. vulgatus is more apparent in Table 18. Despite attempts to select equal numbers of B. vulgatus and fragilis colonies using esculin hydrolysis as the differentiating characteristic virtually all the initially picked colonies resembled B. fragilis. It is also noteworthy that the selected isolates were all anaerobes for the first two intervals (i.e. 24 hours) and then rapidly declined. The organisms isolated after 24 hours still exhibited positive esculin hydrolysis but they were facultative anaerobic organisms. What is particularly relevant here is the rapid die-off of anaerobes between one and two days into the experiment. It appears that B. fragilis is capable of maintaining its viability even in an aerobic environment for 24 hours.

In the two experiments where no pure cultures were seeded into the vessels a comparison can be made between the anaerobes and the coliform viable counts (Figs. 3 and 5). The decline in both these indicator groups establishes that none of them is growing or maintaining itself in the experimental system. The correlation between the declines in the fecal coliforms and the anaerobic counts is similar overall. Total coliform count declines were similar in Exp. I but atypical in Exp. II in that total coliforms slightly increased in numbers from 0-2 days while fecal coliforms declined as expected.

Analyses of the organisms recovered on the Bacteroides selective media from unseeded samples containing raw sewage indicate that the isolates are not, for the most part, Bacteroides. This finding and the percent viability of anaerobes recovered on each of the media suggest these media are neither efficacious nor consistent for the selective isolation of Bacteroides under our described conditions.

The confirmation criteria for the Bacteroides spp. of intestinal origin need to be investigated further. The general grouping we used to define and confirm an isolate as an enteric Bacteroides is Gram-negative, anaerobic rod, esculin hydrolysis positive, resistant to kanamycin (1000 ug) and penicillin-G (2 units), sensitive to erythromycin (60 ug), and capable of growth

on 20% bile (16,29). Using our stock strains of Bacteroides, none of them fit the above grouping in every way. None was capable of growth on 20% bile (Oxgall) medium. B. vulgatus B2-4, a species that is the most prevalent organism in human feces, proved to be esculin negative and penicillin sensitive. The only grouping of criteria which fit our stock strains in toto was Gram-negative obligate anaerobe, resistant to kanamycin and sensitive to erythromycin.

The major differentiating characteristic for the intestinal Bacteroides group is esculin hydrolysis which leads to blackened colonies on appropriate agar media. A number of difficulties became apparent as we investigated this characteristic. The majority of the organisms that exhibit esculin hydrolysis and can grow anaerobically are facultative anaerobes. This potentially high background of organisms in contaminated waters means that whatever selective medium is used must be capable of effectively separating obligate anaerobes from facultative anaerobes. This selection having been accomplished, the esculin hydrolysis then becomes a good differentiating characteristic only so long as the Bacteroides in question are not similar to our B. vulgatus and B. distasonis strains which are esculin hydrolysis negative. In this instance, the esculin positive criterion would miss all the B. vulgatus organisms present and recognize only the remaining prevalent Bacteroides which should theoretically suffice since B. thetaiotamicron is positive and ranked No. 3 in prevalence, and B. distasonis is often esculin positive and ranked No. 4.

The use of either bile salts as a selective or differentiating agent poses some difficulties. It is not apparent from the literature what constitutes bile salts or bile per se. Post, et al. (21) used a bile salt, 0.07% sodium desoxycholate, in their medium for the selective isolation of Bacteroides from sewage. There appeared to be some inhibition of viable recovery because the numbers of Bacteroides did not reflect the numbers in the human intestine. Shimada, et al. (25) compared desoxycholate to Oxgall (Difco) and fresh beef bile. Desoxycholate at 0.1% was found to be inhibitory to B. fragilis whereas 20% Oxgall + 0.1% desoxycholate and 20% fresh beef bile were non-inhibitory or only slightly inhibitory in various media. Furthermore, the action of fresh beef bile was almost the same as Oxgall but somewhat less inhibitory. Subsequently Sutter and Finegold (29) used 20% bile plus 0.1% desoxycholate as a differentiating characteristic for B. fragilis. Vargo, et al. (30) in 1974 made an unsubstantiated statement that 2% Oxgall (Difco) is equivalent to 20% bile and formulated their 20% bile medium with 2% Oxgall. Chan and Porschen (6) referred to the same 20%:2% relationship in the formulation of their Kanamycin-Esculin Bile Agar, the forerunner of our KB medium. Livingston, et al. (16) who proposed BBE for the selection and identification of B. fragilis group referenced Vargo, et al. (30) and used 2% Oxgall. Difco (9) describes its product, Bacto Oxgall, as being equivalent to fresh bile when a 10% solution of Oxgall is attained and thus explains why previous investigators assumed 2% Oxgall is equivalent to 20% fresh bile.

What we can conclude from this discussion is that desoxycholate, a bile salt, is inhibitory for B. fragilis and very different from bile per se. What is not clear is the discrepancy between Shimada, et al. (25) who state

20% Oxgall is equivalent to 20% fresh bile whereas Difco (9) states 2% Oxgall is equivalent to 20% fresh bile. Our results suggest that there is a discrepancy in this area. To recover our organisms from our selective plates we inoculated the colonies into Bacteroides Oxgall Esculin (BOE) liquid medium containing 2% Oxgall because we had observed that our anaerobes and facultative anaerobes were generally stimulated and rarely inhibited. When we tested the growth of our stocks on 20% Oxgall they did not grow when it was expected that they should if 20% Oxgall was equivalent to 20% fresh bile (Table 15).

What is an "unexpected finding" is the effect of 20% bile salts. None of the Bacteroides exhibited growth on 20% bile salts, as would be expected, or 20% Oxgall. Of the enterics only E. aerogenes exhibited growth on 20% bile salts agar. Of the 65 isolates in our experiments, 31 (48%) grew on 20% bile salts, while only one grew on 20% Oxgall. It is possible that inhibition by bile salts would be a negative differentiating characteristic for intestinal Bacteroides species.

The overall results of our study indicate that it is possible to detect intestinal Bacteroides spp. in wastewaters and lake waters seeded with raw sewage and pure cultures of B. fragilis and B. vulgatus using fluorescent antibody staining techniques. Both dFA and iFA procedures can be used satisfactorily for this purpose; however, some problems were encountered with non-specific staining of sample debris, and debris-coating of Bacteroides cells.

Unexpected problems were encountered in the development of an ELISA procedure for the detection of intestinal Bacteroides spp. The primary difficulty was that the filter system needed for a drinking water assay showed high background activity of the assay enzyme system. This problem was due to the non-specific adsorption of the enzyme-tagged anti-Bacteroides serum to the various treated filters tested for the assay.

Intestinal Bacteroides spp. die off more quickly in raw sewage than would be expected based upon studies of pure cultures suspended in lake waters. Thus, the numbers of this genus were always low in raw sewage sampled. Furthermore, there are presently no known criteria which can offer a rapid viable enumeration of the group.

One important finding of the study is that the ratio of viable Bacteroides to fecal coliforms may provide a good indicator of how recently a natural or drinking water supply has been contaminated with raw wastewater. A high ratio of Bacteroides:fecal coliforms would indicate recent contamination whereas a low ratio would suggest earlier contamination. This ratio stems from finding that the rate of die-off of Bacteroides spp. is much more rapid than that of the fecal coliform bacteria.

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